COMPARISON OF TWO PROTEOMIC METHODS FOR ANALYSES OF GLAUCOMA MEDICATION EFFECTS IN VITRO

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NOORA KARISOLA: COMPARISON OF TWO PROTEOMIC METHODS FOR ANALYSES OF GLAUCOMA MEDICATION EFFECTS IN VITRO

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Tämän syventävän työn tarkoituksena oli vertailla kahden eri massaspektrometrimenetelmän, SWATH-MS:n ja iTRAQ:n, eroja silmäsolujen proteomiikan tutkimuksissa. Tutkimustyö oli osa laajempaa kokonaisuutta, jossa tutkittiin glaukoomalääkkeiden vaikutusta silmän pinnan soluihin. Analysoitavina solumalleina käytettiin kahta silmän pinnan solulinjaa, HCE ja NHC. HCE-solulinja on kehitetty ihmisen sarveiskalvon epiteelisoluista, ja NHC-solulinja ihmisen sidekalvon soluista. Solut altistettiin kahdella eri glaukoomalääkkeellä, tafluprostilla ja latanoprostilla, sekä lääkkeiden säilöntäaineella bentsalkoniumkloridilla. Kontrollinäytteinä käytettiin altistamattomia soluja. Altistusten jälkeen kummankin solulinjan näytteet analysoitiin kahdella eri massaspektrometrimenetelmällä, SWATH-MS ja iTRAQ.

Menetelmien välillä vertailtiin niiden tunnistamien proteiinien kokonaismäärää ja tunnistamisen toistettavuutta, pitoisuusmääritysten luotettavuutta ja tulosten yhteneväisyyttä keskenään. SWATH-MS:lla proteiineja tunnistettiin huomattavasti enemmän ja toistettavammin kuin iTRAQ:lla. iTRAQ vaikutti tulosten perusteella tuottavan luotettavampia pitoisuusmäärityksiä kuin SWATH-MS, mutta tulokset analysoitiin pienemmästä määrästä proteiineja. Pitoisuusmääritysten osalta tulokset olivat jokseenkin yhteneviä, mutta tuloksissa ei löytynyt lineaarista suhdetta menetelmien välillä.

Contents

1 INTRODUCTION

1.1 Glaucoma

Glaucoma is a chronic neuropathy of the optic nerve which results in structural changes in optic disc and retinal nerve fiber layer, and causes progressive visual field loss (1-3). There are around 50 different subtypes of glaucoma (1). Primary open-angle glaucoma (POAG) and exfoliation glaucoma are the most common subtypes of glaucoma in Nordic countries (4). Other glaucoma subtypes contain primary angle-closure glaucoma (PACG), and secondary glaucomas e.g. inflammatory glaucoma, pseudoexfoliative glaucoma, pigmentary glaucoma, neovascular glaucoma, and traumatic glaucoma (1). The main risk factors for glaucoma are age, elevated intraocular pressure, ethnic background, myopia, and positive family history (3).

The diagnosis of glaucoma is based on ophthalmoscopy, tonometry, and perimetry (3). Usually structural changes in POAG progress slowly over the years and patients remain asymptomatic in the early stages of disease, while PACG patients may have sudden onset of dramatic symptoms. Especially PACG should be diagnosed and treated properly in the early stages of disease because the structural changes may progress in a short period of time. (1-3) The symptoms of POAG emerging usually in the later stages of disease contain progressively worsening vision (usually unilateral) and difficulty with night vision (1). If left undiagnosed and untreated, glaucoma results in irreversible visual disability and eventually in blindness (1-3,5). Glaucoma is, in fact, the second leading cause of blindness worldwide after cataract (6).

In order to estimate the global prevalence of glaucoma (POAG and PACG) at present and in the future, Tham et al. (2014) examined data from 50 articles in their meta-analysis. In 2013, 64,3 million people aged 40-80 years were estimated to be affected by glaucoma. The global prevalence, thus, was estimated to be 3.54 % among the age-group mentioned above. The prevalence of POAG was 3.05 % while the prevalence of PACG was only 0.50 %. The prevalence of glaucoma among people aged 40-80 years was predicted to increase by 18.3 % in 2020 and by 74 % in 2040, mainly due to aging of the population. Thus, in 2040 112 million people are predicted to be affected by glaucoma. (7)

The objective of the treatment is to prevent visual disability by reducing the risk of development and progression of the structural and functional changes. For the present, lowering the IOP

(intraocular pressure) is the only treatment form for POAG. (2) 30-50 % reduce from baseline in intraocular pressure usually stops the progression of glaucoma changes (3). The treatment modalities involve medical therapies, laser therapies, and surgical managements, of which the medical therapies are usually the first line treatment (3,8). Five different groups of topical medications are currently in use: prostaglandin analogues, beta-adrenergic antagonists, alpha-adrenergic agonists, cholinergic agonists, and carbonic anhydrase inhibitors. In addition, various new drugs are in development for the treatment of POAG including rho kinase inhibitors, adenosine receptor agonists, and modified prostaglandin analogs. (9)

1.2 Prostaglandin analogues in treatment of glaucoma

Topical prostaglandin analogues or beta-adrenergic antagonists are usually the first-line treatment for POAG (8). However, in various studies prostaglandin analogues have showed to be more effective in lowering IOP than beta-adrenergic antagonists (10-15). In addition, prostaglandin analogues lack relevant systemic side effects and are administered only once a daily, thus, prostaglandin analogues are recommended as first-line therapy for glaucoma treatment by European glaucoma society (16,17).

Latanoprost, tafluprost, travoprost, and bimatoprost are currently available for glaucoma treatment in Finland (18). Prostaglandin analogues reduce IOP by increasing aqueous humor outflow in two different ways: by increasing uveoscleral outflow and to a lesser degree by increasing trabecular outflow facility (19). The IOP-lowering effectiveness among different prostaglandin analogues is nearly equivalent, or at any rate the slight differences are not clinically relevant (2,15,20-23). The common adverse effects of prostaglandin analogues encompass iris pigmentation, hypertrichosis of eyelashes, and intraocular inflammation (1).

Long term use of topical ocular medication has been reported to cause inflammatory changes in ocular surface (24). Those changes have suggested to maybe appear as soon as after one year use of topical ocular medication (25). Many topical ocular solutions include preservatives, of which benzalkonium chloride (BAC) is the most used (26). BAC has suggested to be responsible for the allergic, inflammatory, and toxic reactions rather than medicines themselves (24). The adverse effects of BAC have been showed in several in vitro, in vivo, and clinical studies (27-34). BAC has showed to cause cell death already at a concentration 0,0001%. The lethal effect of BAC appears to be dose-dependent: at low concentrations BAC causes cell death via apoptosis, while at high

concentrations cells die via necrosis. (26,31) According to few studies, it appears that prostaglandin analogues may have a protective effect against the adverse effects of BAC (29,31).

1.3 Mass spectrometry in proteomics

Proteomics refers to the large-scale study of protein properties (35). Mass spectrometry is currently a method of choice in proteomics (36). In addition to protein identification, it is able to provide information about various other crucial characteristics including protein-protein interactions, post-translational modifications, and absolute or relative quantification. Quantitative proteomics enables comparative analysis of protein expressions between different biological states, for example disease and healthy state. (37)

1.3.1 Mass spectrometer

Mass spectrometer is a device that measures mass-to-charge (m/z) ratios and relative abundances of ions. There are many different types of mass spectrometers but all of them have in common three main building blocks: an ion source, a mass analyzer, and a detector. The ion source ionizes sample molecules and creates gas phase ions. A mass analyzer, which function is based on electromagnetism, separates ions according to their m/z. The detector converts the energy of incoming particles into a current signal. The signal is registered by the electronic devices and transferred to the computer in the form of mass spectra. There are several different types of ion sources (e.g. gas discharge, thermal ionization, electron ionization, and electrospray ionization), mass analyzers (e.g. time-of-flight (TOF), quadrupole ion trap (QIT), and orbitrap), and detectors (e.g. electron multipliers, faraday detector, and photoplate detector). (38)

Mass spectrometers are able only to detect molecules with charge. This is because molecules with charge are responsive for electromagnetic fields which are used in mass analysis. (36) Different ion types can be formed depending on the molecules and the nature of the ionization process (39). Peptides are commonly positive in charge because of the sample preparation using most commonly trypsin which creates peptides usually containing at least two positive charges, one at the N-terminus and another at the C-terminal lysine or arginine (40). In addition, ions can be produced also by attracting one or more electrons or even a charge carrying atom or group. However, negative and positive ions can’t be detected simultaneously due to polarity of the voltage applied to

the detector which is opposite to the ions attracting to the detector. (39) In this study we have only used positive charge in peptide analysis.

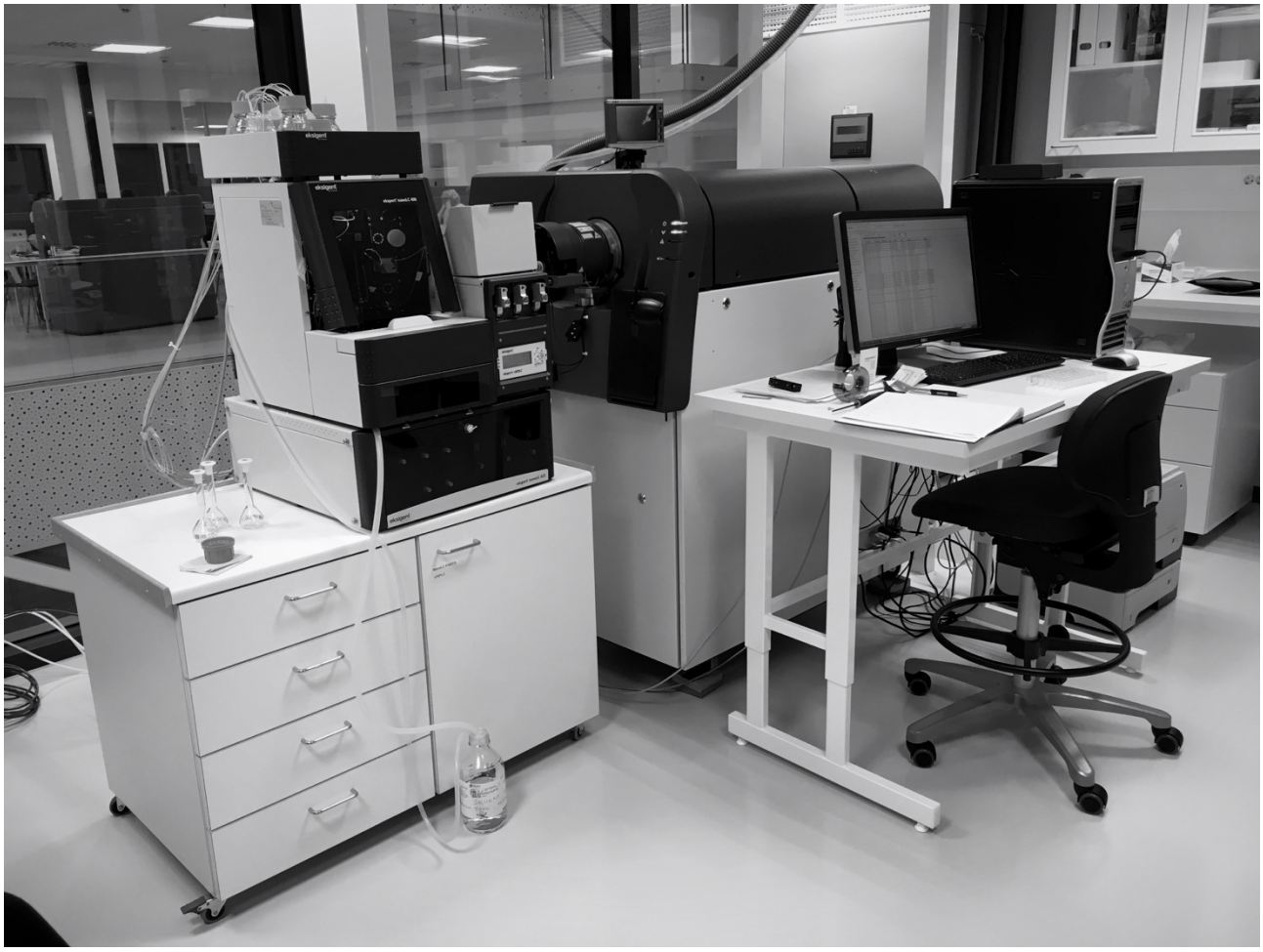

Figure 1. AB Sciex NanoLC 425 coupled to MSTOF mass spectrometer which was used in this study.

1.3.2 Separation methods

Samples with high dynamic range (e.g. biological fluids) cause extra challenge for a complete and reproducible analysis of protein content. Very abundant proteins may give rise to suppression effect that can complicate or preclude the detection of low abundant proteins. Hence, fractionation of proteins prior to mass spectrometry analysis is required in order to detect all proteins across the dynamic range of a sample. (41) Consequently, in order to increase sensitivity, dynamic range, and selectivity, several different separation methods (online or offline) have been applied in conjunction with the main building blocks of mass spectrometer. Chromatography and electric-field driven separations are such methods. (38)

Separation of sample molecules by chromatography is based on the different physiochemical properties between the molecules. A chromatographic system consists of a mobile phase and a stationary phase. A sample is dissolved in the mobile phase which then carries the sample through the stationary phase. The molecules of the sample have different affinities for the stationary phase and therefore travel through the stationary phase at different speeds (i.e. have different retention times). The two main types of chromatography are liquid chromatography in which the mobile phase is liquid, and gas chromatography in which mobile phase is gaseous. (38) In this study we used liquid chromatography (AB Sciex NanoLC 425) coupled to MSTOF mass spectrometer (*Figure 1*).

1.4 SWATH-MS

SWATH-MS (sequential windowed acquisition of all theoretical fragment ion mass spectra) is a label-free mass spectrometric technique for qualitative and quantitative protein determination. The method was first published in 2012 by Gillet et al. SWATH-MS combines data-independent acquisition method (DIA) with a targeted data extraction strategy and provides reproducible and accurate quantification with high identification rate. (42) *Figure 2* illustrates the workflow of SWATH-MS.

In SWATH-MS, the mass spectrometer repeatedly cycles through usually 25Da wide (can be adjusted) isolation windows, “swaths”, across the mass range in interest (generally 400-1200m/z) (42). With DIA mode, all precursor ions that fall into the isolation window are fragmented simultaneously and spectra of all fragment ions is acquired (43). During the entire chromatographic separation, the same precursor isolation window is fragmented over and over again. Hence, merely by one single injection, this acquisition method is able to generate a complex fragment ion map that is continuous in both fragment ion intensity and retention time dimensions. (42,43)

After data acquisition, the peptides can be identified and quantified by targeted data extraction strategy. Identification and quantification are both performed from MS2 spectra. SWATH-MS can be applied only on a fast, high resolution MS instrument in order to achieve highly specific fragment ion data of all the precursor ions. (42) Currently SWATH-MS is available for various LC-MS/MS devices.

1.4.1 Spectral libraries

The data analysis in SWATH-MS quantification requires spectral libraries that have been generated beforehand (42). Fragment ion maps generated by data acquisition are compared to spectral libraries in order to find matches (44). Spectral libraries contain information about peptide characteristics (e.g. fragment ion signals, their relative intensities and chromatographic concurrence) which is utilized in peptide identification. The peak areas and intensities of identified peptides are used to assess the relative abundances. (42)

Shotgun proteomics is a mass spectrometric method that allows identification of at least thousands of proteins from a sample, and thus is usually utilized in making of spectral library, which is also used in iTRAQ method. In shotgun proteomics, mass spectrometer uses data-dependent acquisition mode (DDA), in which a number of the most abundant peptides in given chromatographic elution time are selected to fragmentation and recorded in MS scan. (44) Building a library starts with the selection of representative samples that are then analyzed with DDA method preferably with the same instrument than subsequent SWATH-MS analyses (45).

Alternatively, the fragment ion spectra can be built from synthetic peptides or recombinant proteins or they can be computationally predicted (45-47). After acquisition, the DDA data will be processed and searched against protein sequence database. Finally, consensus spectral library will be built from confidently assigned spectra, and SWATH-MS spectral library will be generated from it by selecting the most intense fragment ions for each precursor. (45) Instead of building a spectral library, nowadays some proteome-wide spectral libraries are completed and publicly available for SWATH-MS analysis. Recently a library containing over 10,000 human proteins were developed and optimized for SWATH-MS. (43,45,48-50)

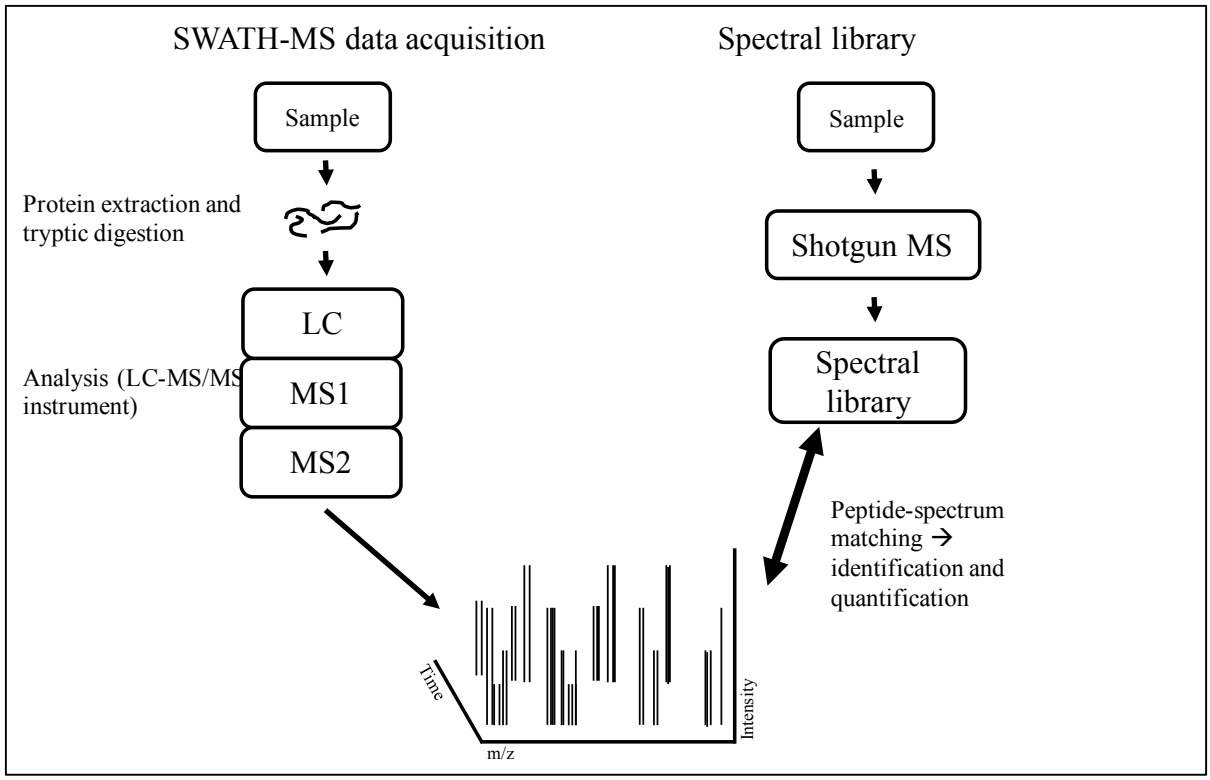

Figure 2. The workflow of the SWATH-MS. First, proteins in a sample are extracted and enzymatically digested. The sample is then analyzed by LC-MS/MS mass spectrometer. The outcome is a complex fragment ion map which is continuous in three dimensions: time, m/z, and peak intensity. In order to identify and quantify proteins, the fragment ion map is matched with spectral library that has been generated beforehand e.g. using shotgun proteomics.

1.4.2 The pros and cons of the SWATH-MS

Selected reaction monitoring (SRM) is considered the gold standard of proteomic quantification methods (42). However, SRM requires targeted proteins to be determined prior to data acquisition and is able to quantify approximately 50-100 proteins in a single run without compromising identification and quantification accuracy (51). SWATH-MS provides greatly higher throughput than SRM with possibility to identify and quantify at least thousands of proteins (*Table 1*). First of all, high throughput is due to DIA method which acquires data independently on a priori protein determination. Second, permanent digital records of SWATH-MS data allow reanalysis with more complete spectral libraries and thus enables higher throughput. (42,43)

Moreover, it has been showed that SWATH-MS is sensitive enough to identify and quantify peptides down to the hundred amol range which is a little poorer than sensitivity of SRM (42). However, the performance of SWATH-MS depends vitally on coverage and quality of spectral

libraries. Thus, the ideal library would contain at least all peptides in interest or even whole proteome. (45)

Other performance characteristics such as dynamic range, reproducibility, and accuracy of quantification has proved to be comparable with SRM (42,44,51,52). SWATH-MS is able to quantify peptides reproducibly at least over a dynamic range of 4 orders of magnitude (42,52). The reproducibility of quantification is usually estimated via coefficient of variation (CV). Coefficient of variation denotes the ratio of standard deviation to mean. In SWATH-MS experiments, the CVs have been relatively small referring to high reproducibility of quantification. The quantification has also discovered to produce accurate measurements. (*Table 1*) In two studies, a small ratio compression effect has been reported with SWATH-MS (44,53). Samples with higher protein loads and larger fold changes increased the underestimation effect (44). However, in general the reproducibility and accuracy have thought to remain high from simple samples to more complex samples, even in whole cell proteome complexity level (42,44,52).

Table 1. Studies that evaluate the performance characteristics of SWATH-MS.

Study	Sample	LC-MS/MS set up	Number of identifications	Reproducibility	Accuracy of quantification
Huang et al. (2015) (44)	Mouse fibrosarcoma cells	TripleTOF™ 5600+ + Eksigent 425 nano 2D HPLC	In total 3600 proteins across four samples (and three technical replicates of each) which contained different peptide mixture loads (0,25µg, 0,5µg, 1µg, 2µg). More proteins were identified when more peptide mixture was loaded: 410 proteins more were identified when peptide mixture load increased from 0,25µg to 2µg.	CV%s were determined on transition ion, peptide, and protein levels. Quantification variance was smaller on samples with higher protein amounts. For the sample containing 2µg peptide mixture: CV% < 10% for 84% of the proteins and CV% < 20% for 91% of the proteins. CV < 20% for 88%, 83%, and 78% of the proteins (1µg, 0,5µg, and 0,25µg respectively).	Samples with different protein loads and theoretical protein ratios (1:2, 1:4 and 1:8) were quantified: all measured ratios were smaller than theoretical ratios. Samples with higher protein loads and larger fold changes were underestimated the most. However, measurements with theoretical 1:1 ratio showed very high quantification precision and accuracy.
Bourassa et al. (2015) (53)	Duodenal tissue biopsies	TripleTOF 5600, Agilent 1200 HPLC	A total of 2,290 proteins.		Bovine serum albumin was spiked into samples at 1:50 ratio. Ratio values from experiment were 29,5 +/- 5 thus indicating a small compression effect.
Selevsek et al. (2015) (52)	Saccharomyces cerevisiae -yeast	TripleTOF™ 5600 + NanoLC-2Dplus HPLC system (Eksigent)	2578 proteins were detected confidently in single run. In further examinasions of reproducibility of identification, 2880+-7 proteins were identified in single run, and 80% of those were detected in all four runs and more than 90% were detected in three of four runs.	CV% of the integrated transition peak areas across four runs were determined (included only peptides detected in all runs): CV% ≤ 10% for 76% of the assays and CV% ≤ 40% for 96% of the assays. CVs were comparable between low and high signal intensities.	Mixtures containing two differentially labeled tryptic digests in 1:1 ratio and 1:10 ratio were quantified: fold changes were 0,92±0,14 for 1:1 ratio and 10,47±4 for 1:10 ratio.
Zhang et al. (2014) (84)	Two non-small-cell lung cancer cell lines	Triple TOF 5600, NanoLC-2D Ultra	A total of 824 proteins were identified. 562 of those were quantified repeatedly in three technical replicates.		
Gillet et al. (2012) (42)	Saccharomyces cerevisiae -yeast	TripleTOF™ 5600 and Eksigent 1D+ Nano LC system (reproducibility experiment) or Eksigent Nano-LC-2D+ + nanoFlex cHiPLC system (quantification accuracy experiment)		CV% was determined from 23 peptides which were spiked at constant consentrations into samples containing yeast trypsin digest as proteomic background: 13.7%.	The experiment was performed as in (Picotti, P. et al. 2009). Samples were tryptic digests of a mixture of 1) lysate of yeast cells that sampled throughout the metabolic shift from fermentation and respiration and 2) 15N-labeled yeast lysate background (internal standard for the fold change calculations). Quantification accuracy achieved the same level than accuracy of SRM.

1.5 iTRAQ

iTRAQ (isobaric tags for relative or absolute quantitation) is a mass spectrometric technique used widely in quantitative proteomics (54). It was developed by Applied Biosystems (nowadays Sciex) in early 2000. iTRAQ utilizes isobaric tagging reagents enabling simultaneous identification and quantification of proteins using LC-MS/MS (37,55). *Figure 4* illustrates the workflow of the iTRAQ.

The iTRAQ reagents were originally designed for simultaneous analysis up to 4 different biological samples but are nowadays available also as an 8-plex kit and 12-plex kit (55,56). The reagents consist of a peptide-reactive group (NHS ester) and an isobaric tag, that consist of a reporter group (N-methylpiperazine) and a mass balance group (carbonyl) (*Figure 3*). The isobaric tags differ in isotopic compositions of oxygen, nitrogen and carbon. (55) The reporter group masses for 4-plex

range from m/z 114.1 to m/z 117.1, and for 8-plex masses are 113.1, 118.1, 119.1 and 121.1 (54). The balance group also ranges in mass, thus keeping the combined mass of tag identical across different tags (55). Reporter ion masses have been designed carefully in order to avoid interference by ordinary peptide fragments, thus m/z 112 and 120 were excluded as reporter ions for 8-plex kit because arginine and phenylalanine have the same mass (56,57). The peptide-reactive group specifically reacts with primary amines of peptides and forms an amide linkage to N-terminal of peptide or Ɛ-amino group of the lysine side-chain (55). Hence, almost all peptides that exist in a sample are labeled and can be identified and quantified (37).

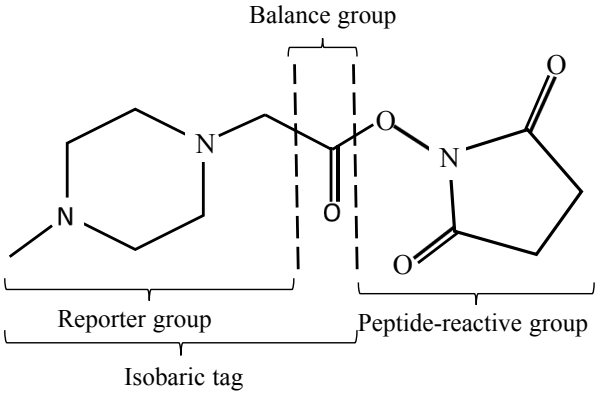

Figure 3. Based on a figure by Ross et al. (2004). iTRAQ reagents consist of a peptide-reactive group and an isobaric tag which includes a reporter group and a mass balance group. The peptide-reactive group reacts with primary amines of peptides while reporter groups are used to assess quantities of peptides. The function of the balance group is to keep the mass constant across different tags. The tags differ in isotopic compositions of oxygen, nitrogen, and carbon. (55)

After labeling step, samples are pooled and analyzed by LC-MS/MS (55). Every identical peptide from each sample appears as a single peak in the MS1 spectrum because of the isobaric nature of the reagents. During further fragmentation in MS/MS, there is a neutral loss of balance group and reporter group ions are released yielding ions at different m/z. The intensities of reporter ion peaks are directly proportional to the relative abundances of peptides before labeling. In addition to reporter ion peaks, series of y- and b-ions are produced, and can be used for identification or sequence confirmation. (37)

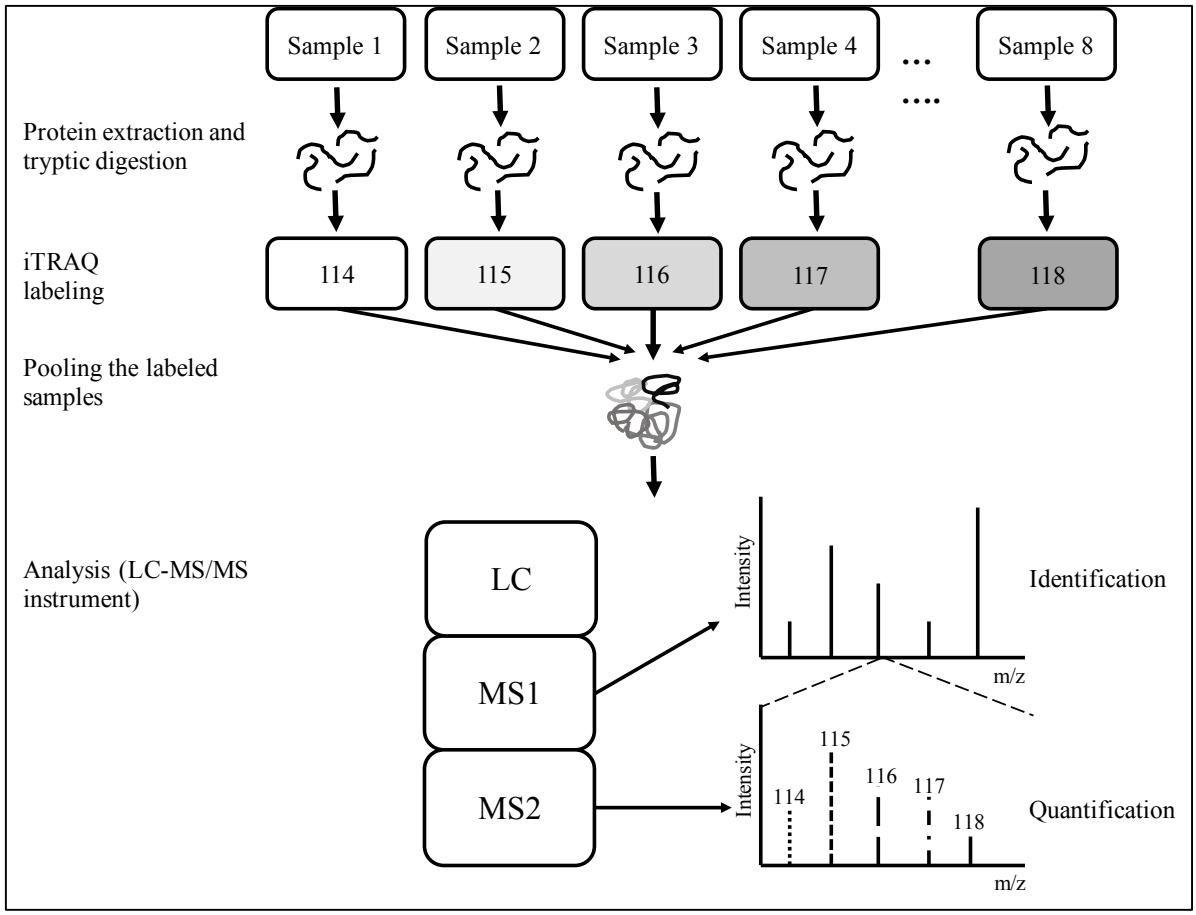

Figure 4. The workflow of the iTRAQ. Proteins from samples are first extracted and enzymatically digested. Following digestion, peptides are labeled with iTRAQ labeling kit and pooled. Up to twelve samples can be labeled and analyzed simultaneously. Pooled samples are analyzed using LC-MS/MS instrument. Identical peptides from all samples appear as a single peak in MS1 spectrum, from which identifying is performed. After further fragmentation during MS2, iTRAQ reporter group ions are released. Reporter group ions from different samples appear as different peaks in MS2 spectrum, from which quantifying can be performed.

iTRAQ labeled samples can be analyzed by variety of MS/MS platforms: Q-TOFs, TOF-TOFs, Ion traps, and Orbitraps (55,58-60). At the beginning, ion traps suffered from the poor capability to detect the low m/z reporter ions: fragment ions with m/z values less than 25-30% of the precursor ion m/z values were not detected in MS2 spectrum (61). However, several methods, including pulsed Q dissociation (PQD), higher-energy C-trap dissociation (HCD), and combined collision-induced dissociation (CID) and HCD, have been developed to overcome this limitation (58,62,63). Recently, comparison of LTQ Orbitrap Velos, 4800 MALDI-TOF/TOF, and 6530 Q-TOF showed that the identification rate was highest with Orbitrap which identified approximately four times more proteins than the other instruments did. Further, Orbitrap and MALDI proved to be the most

trustworthy with respect to quantification (bias and variance were evaluated), whereas QTOF showed poorer results. (64)

1.5.1 The pros and cons of the iTRAQ

At its best, iTRAQ is able to reliably identify up to thousands of proteins. However, protein identification rate varies largely between different experimental settings and LC-MS/MS instrumentation. (*Table 2*)

Concerns about accuracy of quantification have been arisen. Accuracy refers to how much the observed values differ from the true values, and is affected by systematic bias. Underestimation of fold changes of protein expressions has proved to be the main challenge with iTRAQ, and has been reported by several studies. (54,59,64-66) It has been named ratio compression as it arises from iTRAQ's tendency to compress ratios towards 1, thus reporting the observed fold changes smaller than the true fold changes are (37). Ratio compression has seen to become more pronounced with larger fold changes (65,66). However, the trends of up- or down-expression should be correct (54). Ratio compression has been observed across different mass spectrometric instrumentations and experiments, thus showing to be independent on the LC-MS/MS instrumentation (*Table 2*). The fold changes smaller than twofold have been concluded to be difficult to detect (66).

Cross-label isotopic impurity is one cause leading to ratio compression. Impurities arise mainly during manufacture and are expected. Hence, the effect of isotopic impurities can be corrected during analysis. Manufacturer commonly provides correction information with regard to the 4-plex. The correction of the effect of isotopic impurities for 8-plex is more difficult. The label-dependent percentage contamination must be determined by MS/MS and once values are known, correction algorithms can be applied to reduce underestimation. (54)

More serious factor affecting ratio compression is the background contamination that arises from interfering ions (59,65). When precursor ion is selected for fragmentation during MS/MS, also one or more, co-eluting precursor ions with similar m/z, are selected as well. Therefore, the precursor ion in demand and spare precursor ions are sequenced and quantified at the same time. This raises reporter ion intensities and results in compression of the ratio. (54) The result is mixed MS/MS spectra which impairs accuracy of fold change determinations (54,59). In addition, because mixed MS/MS spectra contain unidentifiable fragment ions, they also impair identification rate in large-scale data sets (67). This co-isolation is proportional to sample complexity (59).

Several studies have tried to find a solution for background contamination issues. Narrowing the MS/MS isolation width is one approach to reduce the effect of background contamination. In theory, with narrower isolation window fewer interfering ions are co-selected and co-fragmented. (59,65) Bantscheff et al. (2008) performed four LC-MS/MS runs with different precursor ion isolation widths. The observed fold changes were significantly lower than the expected ratio in all four cases. At narrow isolation widths the ratio were closest to the expected value, thus showing that the presence of increasing amounts of nearly isobaric peptides significantly contribute to the total reporter ion intensities. (59) However, Karp et al. (2010) investigated three different isolation window widths (2-fold increase in the contamination) and observed no statistically significant difference between settings suggesting that other factors have more effect on background contamination than isolation window width (65). In addition, narrowing the isolation window decreases the signal intensity and results in poorer limit of quantification (59).

Ow et al. (2011) focused on minimizing sample complexity by effective fractionation prior to MS/MS. They observed that instead of using a typical low-/medium-resolution fractionation technique, using a high-resolution fractionation technique (HILIC) reduces the ratio compression. Based on their results the accuracy can be improved approximately 20% with high-resolution fractionation technique. (68)

Few studies have tried to eliminate issues through different modifications on the data acquisition stage (69-71). Wenger et al. (2011) developed a method called QuantMode in which they applied gas-phase purification method. Proton-transfer ion-ion reactions reduce ion charge state thus changing m/z values of ions. This results in purified precursor ion population for further fragmentation and provides improved accuracy and precision. (70) This method was originally designed for high-resolution QLT-Orbitrap hybrid systems, but recently, Vincent et al. (2013) extended this method to encompass low resolution devices too. (70,71)

Ting et al. (2011) applied triple mass spectrometry (MS^3) in order to enhance the ion selection specificity. The most intense MS2 fragment ion was selected for M3 stage and used to provide quantitative data. This method improved both accuracy and precision by almost completely eliminating the adverse effect of interfering ions. Unfortunately, also with this method 12% decrease in quantified proteins was reported, and it can be applied only for ion trap instruments. (69)

Karp et al. (2010) proposed a method to address accuracy problem in data analysis stage. They observed a linear relationship between the expected and the observed ratios, and proposed that readings of known proteins that span through the range of expected ratios can be used to calculate a single correction factor. For complex samples in which linear relationship is thought to be disturbed they proposed development of a kit that consists of a mixture of proteins at known ratios. This mixture would be added to samples before iTRAQ labeling, and after acquisition proteins at known ratios would be used to calculate the correction factor. (65)

In addition to concerns of accuracy of quantification, also concerns about precision has been arisen. Precision refers to how reproducible the repeated measurements are, and is affected by random errors. (65,66) On one hand, it depends on the quality of individual mass spectrum, and on the other hand, it depends on the number of available spectra for quantification (59). The precision issues are independent on LC-MS/MS instrumentation, labeling kit (4-plex or 8-plex), and analytical packages (65). Precision has been found to be the function of mean abundance: variance is higher for low intensity signals than for high intensity signals (58,59,65,66,72,73). This is significant problem because low intensity signals dominate the data sets, and in biological studies low intensity peptides may be among the most interesting peptides (65).

Several statistical analysis methods have been proposed to minimize variation and thus address precision issue. Such methods are for example outlier removal, weighted means, inclusion filters, and logarithmic transformation. (65,74-76) However, according to Karp et al. (2010), these methods discovered not to be able to address the precision issue while maintaining sensitivity.
Karp et al. (2010) applied a variance stabilizing transformation to address the precision issue. This method stabilizes variance across all intensity ranges and simplifies the downstream analysis. In addition, it allows using low intensity readings which is necessary with biological samples. However, there is a drawback: the ratios of small peak areas are compressed towards one thus making underestimation issue more pronounced. (65)

Recently, Mahoney et al. (2011) recommended weighted least squares (WLS) differential expression model over the VSN. In WLS a weight is given to each abundance value: the peptides that have been measured with less variance are given more weight and the peptides that have been measured with greater variance are given less weight. (66)

Table 2. Studies that evaluate the performance characteristics of iTRAQ.

Study	Sample	LC-MS/MS set up	Number of identifications	Reproducibility	Accuracy of quantification
Bourassa et al. (2015) (53)	Duodenal tissue biopsies	TripleTOF 5600, Agilent 1200 HPLC	A total of 3,886 proteins in at least one replicate.		The compression effect was seen in linear regression model, volcano plot and density plot.
Zhang et al. (2014) (84)	Two non-small-cell lung cancer cell lines	Triple TOF 5600, NanoLC-2D Ultra	966 and 995 proteins were identified in two technical replicates. 636 of those were quantified repeatedly in two technical replicates.		
Hultin-Rosenberg et al. (2013) (64)	Lung cancer cell line A549	1200 nano-LC + LTQ Orbitrap Velos or Ultimate 3000 LC + 4800 MALDI-TOF/TOF or 1200 nano-LC + 6350 QTOF	Orbitrap 2,453 proteins, MALDI 620 proteins, QTOF 238 proteins.	The relative standard deviation (RSD) per peptide was calculated across the eight iTRAQ channels. RSD was considerably higher for the peptides with low intensity.	The fold change for all peptides was calculated and related to minimum peptide signal intensity. The fold change compression toward one was seen idependent on intensity level. The fold changes were approximately 5% lower than the expected fold change.
Mahoney et al. (2011) (66)	Saccharomyces cerevisiae -yeast as background, 16 non-yeast spike-in proteins	LTQ Orbitrap or MALDI 4800 TOF/TOF	In total 1449 proteins from 3 studies (ten samples) were identified. 99% (1436) of the proteins was identified in study 1 (four samples, Orbitrap), 35% (512) of the proteins was identified in study 2 (four samples, MALDI), and 22% (312) of the proteins was identified in study 3 (two samples, MALDI). 19% (273) of the proteins was identified in all three studies.	Median CV% were calculated for each study: for study 1, 2, and 3 CV%s were 25%, 12%, and 17% respectively. In all three studies CV% was higher for peptides with lower abundance.	The expected fold changes of 16 non-yeast proteins were compared to the observed fold changes: the observed fold changes were underestimated. The bias increased as the expected fold changes increased but was independent on molecular weight and the amount of protein spiked into the mixture.
Karp et al. (2010) (65)	Erwinia, proteins at known ratios (BSA, Cytochrome c, Enolase, Phosphorylase b), yeast grown under nutritionally limiting conditions	QSTAR or LTQ-OrbitrapXL	Number of identifications were calculated from samples that contained yeast grown under nutritionally limited conditions. Variance-stabilizing transformated data yielded 923-1,042 identified proteins in one sample. Log-transformed data yielded 923-1,040 identified proteins in one sample.	An aliquot of the same sample was labelled by each isobaric tag and then combined before data acquisition by LC MS/MS. Different sample types, MS/MS systems and both, 4-plex and 8-plex tags were used. The variance observed to be more pronounced for low intensity peptides than for high intensity peptides. This effect was irrespective of iTRAQ labeling kit, LC-MS/MS instrumentation, and analytical packages.	Four proteins at known ratios (up to fourfold) were spiked in unchanging amount of background. Systematic ratio underestimation was observed, and there was a linear relationship between the expected and the observed ratios. Underestimation became more obvious for larger fold changes and was independent on LC-MS/MS instrumentation.

1.5.2 4-plex versus 8-plex

Few studies have compared different labeling kits of iTRAQ (77-79). Pichler et al. (2010) compared iTRAQ 4-plex, TMT 6-plex and iTRAQ 8-plex with respect to the numbers of identified peptides and proteins in shotgun proteomics experiments. They used an LTQ Orbitrap mass spectrometer, a hybrid CID-HCD method, and Proteome Discoverer software for data acquisition and analysis. Study was performed with both a mixture of complex biological sample (HeLa cells lysates), and a mixture of standard proteins in defined ratios. They observed that in comparison to 4-plex the number of unique peptides was more than 70% lower and the number of proteins was more than 60% lower with iTRAQ 8-plex labeling for both the standard proteins and the complex biological sample. They considered that the different peptide identification rates result probably from several factors. (77)

In contrast, Pottiez et al. (2012) found divergent results in their study using human plasma as a highly complex sample. Data acquisition and analysis was performed by using ABsciex 4800

MALDI-TOF/TOF mass spectrometer and ProteinPilot 4.0 software. First, they observed that peptide labeling with 8-plex provides better accuracy of quantitation compared to 4-plex without compromising on peptide or protein identification. Identification rates for proteins and peptides were slightly lower for 8-plex compared to 4-plex but the differences were not significant. Second, they observed that when confidence of protein identification decreases, the ratios for individual proteins dispersed in both instances. This impact was, however, lesser for 8-plex tags. (78)

Unlike the previous two studies which used peptide-level labeling, Nie et al. (2013) applied protein-level labeling strategy to study performance between iTRAQ 4-plex, iTRAQ 8-plex, and TMT-6. They studied serum glycoproteins by using Orbitrap Elite mass spectrometer and Proteome Discoverer software for data acquisition and analysis. In agreement with Pichler et al. (2010), they also observed that peptide and protein identification rates were highest with iTRAQ 4-plex when compared to TMT 6-plex and iTRAQ 8-plex: 20% fewer proteins were identified and quantified using 4-plex kit compared to 8-plex kit. TMT-6 and iTRAQ 8-plex showed to have similar performance in contrast with the results of Pichler et al. (2010). (79)

1.6 The aim of this study

For clinical purposes, one main target is to identify proteins as specific biomarkers for diseases. Those biomarker proteins vary in abundance between healthy and disease states, and in some cases they may exist only in either state. Specific biomarkers will help in understanding underlying pathogenic mechanisms of diseases, in discovering early and sensitive diagnostic tools, in identifying therapeutic targets, and in predicting of therapeutic outcome. (37,41)

In this study, the aim was to investigate the performance characteristics of two mass spectrometric methods for *in vitro* proteomic studies of conjunctival and corneal cells. This study was part of a larger entity in which we studied the effects of glaucoma medication to the conjunctival and corneal cells of the eye. Two cell lines, NHC and HCE cells, were used as analyzed samples. The cells were exposed to two glaucoma medicines, tafluprost and latanoprost, and in addition, to preservative benzalkonium chloride (BAC). Unexposed cells were used as a control samples. After exposures, the samples were analyzed by two different mass spectrometric methods, SWATH-MS and iTRAQ. My part in this study was to process the SWATH-MS samples after exposures and prior to mass spectrometric analysis, and to compare the results from iTRAQ and SWATH-MS analyses.

2 METHODS

2.1 Cell lines and exposures

In this study we used two immortalized cell lines: human corneal epithelial (HCE) cells and normal human conjunctival (IOBA-NHC) cells. HCE cell line has been produced by immortalizing human corneal epithelial cells using a recombinant SV40-adenovirus vector (80), whereas IOBA-NHC cell line has been produced via spontaneous immortalization of normal human conjunctival cells growing in culture medium (81). Both cell lines show morphologic and functional properties of normal corneal and conjunctival epithelial cells, being suitable for *in vitro* experiments (80,81).

Both cell lines were exposed to benzalkonium chloride (BAC), tafluprost (Taflotan®, Santen Oy), and latanoprost (Xalatan®, Pfizer). BAC is the most commonly used preservative in topical ophthalmic medications (82). Taflotan and Xalatan are prostaglandin analogs which are used in treatment of open angle glaucoma. Taflotan is unpreserved preparation while Xalatan contains 0,02 % BAC as a preservative. After 24h drug exposures, cells were collected for proteomic analysis. Unexposed cells were used as a control.

2.2 Sample processing

2.2.1 Cell lysis and protein extraction

SWATH-MS and iTRAQ samples: To lyse the cells, 100 µl Pierce® RIPA lysis buffer (Thermo Fisher Scientific, San Jose, CA, USA) supplemented with 0.1 % HALT™ protease inhibitor cocktail (Thermo Fisher Scientific, San Jose, CA, USA) was added onto cells / well and samples were dissociated using a pestle. The samples were then mixed with a vortex for 2 min, followed by incubation first in an ultrasonic bath for 5 min, and then on ice for 25 min. Samples were then centrifuged at 14,800 rpm for 15 min at 4 °C, and supernatants were transferred to clean tubes.

DC Protein Assay kit II (Bio-Rad, Hercules, CA, USA) was used to determine protein concentrations. 60 µg of protein was precipitated by adding cold acetone and incubating overnight

at -20 °C. The following day, samples were centrifuged at 14,000 rpm at 4 °C for 15 min, the acetone was decanted and samples were left to dry.

2.2.2 Protein digestion

SWATH-MS and iTRAQ samples: Samples were resuspended in 50mM ABC (ammonium bicarbonate solution, Sigma Aldrich, St. Louis, MO, USA), 2 % SDS (sodium dodecyl sulfate, Sigma Aldrich, St. Louis, MO, USA) solution, mixed with a vortex for 2 min, and centrifuged at 14,000 rpm for 1 min. In order to reduce disulfide bonds, first 50mM TCEP (Tris-(2-carboxyethyl)-phosphine, Sigma Aldrich, St. Louis, MO, USA) was added, mixed with a vortex for 2 min and centrifuged at 14,000 rpm for 1 min. Then, sample tubes were incubated in interval mixing (15 min at 1,250 rpm and 1 min stable) at 60 ˚C for 60 min, followed by centrifugation at 14,000 rpm for 1 min. Protein digestion steps were performed using FASP™ Protein Digestion Kit (Expedeon, Cabmbridgeshire, UK): 75 % urea solution was added to Spin Filters and samples were transferred to them, followed by centrifugation at 14,000 g for 15 min. Urea solution was added again to the Spin Filter and centrifuged. Reconstitution of disulfide bonds was prevented by adding iodoacetamide and urea solutions to the Spin Filter and incubating in darkness for 20 min. After incubation, samples were centrifuged at 14,000 g for 10 min. Next, samples were washed three times with urea solution, followed by centrifugation at 14,000 g for 10 min after each wash. Same washing steps were then performed with ABC for three times. Trypsin solution (AB Sciex, Framingham, MA, USA) was added at 1:25 (trypsin:protein) ratio and samples were incubated in interval mixing (15 min at 1,250 rpm and 5 min stable) at 37 ˚C for 16 hours, followed by cooling into 4 ˚C and centrifugation at 14,000 rpm for 1 min. After that, samples were washed two times with ABC and centrifuged at 14,000 rpm for 10 min after each wash. Finally, NaCl (sodium chloride, FASPkit) was added, followed by centrifugation at 14,000 rpm for 15 min and drying in vacuum concentrator until there were left approximately 100 µl.

2.2.3 Desalting

SWATH-MS and iTRAQ samples: Samples were dissolved into 0,1 % TFA (trifluoroacetic acid, Sigma Aldrich, St. Louis, MO, USA). The sample clean up tips (Pierce C18 tips, Thermo Fisher Scientific, San Jose, CA, USA) were flushed with 50 % ACN (acetonitrile, Sigma Aldrich, St. Louis, MO, USA) twice and with 0,1 % TFA twice. Sample solutions were flushed in the tips 5-10

times. After that, the tips were washed with 2,5 % ACN + 0,1 % TFA solution twice. Samples were then transferred into clean tubes with solution containing 80 % ACN + 0,1 % FA (formic acid, Sigma Aldrich, St. Louis, MO, USA). Finally, samples were dried in vacuum concentrator.

2.2.4 iTRAQ labeling

iTRAQ reagents were dissolved into ethanol and processed according to manufacturer's instructions (AB Sciex, Framingham, MA, USA). Samples were dissolved into iTRAQ dissolution buffer and labeled with the iTRAQ reagents as shown in *Figures 5 and 6*: HCE-Control 1 & 2 & 3 with 121, 114 and 121 tags respectively, HCE-BAC 1 & 2 & 3 with 117, 119 and 117 tags respectively, HCE-Taflotan 1 & 2 & 3 with 115, 117 and 115 tags respectively, HCE-Xalatan 1 & 2 & 3 with 113, 115 and 113 tags respectively, NHC-Control 1 & 2 & 3 with 118, 116 and 114 tags respectively, NHC-BAC 1 & 2 & 3 with 115, 113 and 119 tags respectively, NHC-Taflotan 1 & 2 & 3 with 113, 119 and 117 tags respectively, and NHC-Xalatan 1 & 2 & 3 with 119, 117 and 115 tags respectively. The labeled samples were incubated in interval mixing (15 min at 1,200 rpm and 1 min stable) at room temperature for two hours, followed by centrifugation at 13,500 rpm for 5 min. Finally, samples were pooled together as indicated in *Figures 5 and 6* and dried by vacuum concentrator for approximately 1,5 hours.

2.2.5 Desalting of iTRAQ samples

Desalting of labelled iTRAQ samples was performed using UltaMicroSpin Columns (Nest Group inc., Southbro, MA, USA) according to manufacturer's instructions: The column filters were conditioned by adding ACN into each filter, followed by centrifugation at 800 rpm for 30 s. This step was repeated once. After that, 0,1M TEAB (triethylammonium bicarbonate, Sigma Aldrich, St. Louis, MO, USA) was added into each filter and centrifuged at 1,000 rpm for 30 s. This step was also repeated once. New collection tubes were transferred under the spin columns and sample was added into necessary amount of spin columns (up to 30µg or 100µl per column). Samples were centrifuged at 1,000 rpm for 30s. Filtrates were transferred back to spin columns and centrifuged at 1,000 rpm for 30 s. Spin columns were washed twice with 0,1M TEAB after which 80 % ACN + 0,1 % FA was added, followed by centrifugation at 1,200 rpm for 1 min and repeated once. Sample was desalted altogether three times with same column. All the final elution solutions were collected into same tube and dried in vacuum concentrator.

2.2.6 Sample reconstitution

Finally, both SWATH-MS and iTRAQ samples were dissolved in 0,1 % FA + 2 % ACN.

2.3 Mass spectrometry analysis

iTRAQ analysis was performed as described earlier (83): Digested peptides were analyzed by Nano-RPLC-TripleTOF instrumentation using Eksigent 425 NanoLC coupled to high speed TripleTOF™ 5600+ mass spectometer (Sciex, Concord, Canada). A microcapillary RP-LC column (cHiPLC® ChromXP C18-CL, 3 µm particle size, 120 Å, 75 µm i.d × 15 cm, Eksigent Concord, Canada) was used for LC separation of peptides. Samples were first loaded into trap column (cHiPLC® ChromXP C18-CL, 3 µm particle size, 120 Å, 75 µm i.d × 5 mm) from autosampler and flushed for 10 min at 2 µl/min (2 % ACN, 0.1 % FA). The flush system was then switched to line with analytical column. Cell samples were analyzed with 120 min 6 step gradient using eluent A: 0.1 % FA in 1 % ACN and eluent B: 0.1 % FA in ACN (eluent B from 5 % to 7 % over 2 min, 7 % to 24 % over 55 min, 24 % to 40 % over 29 min, 40 % to 60 % over 6 min, 60 % to 90 % over 2 min and kept at 90 % for 15 min, 90 % to 5 % over 0.1 min and kept at 5 % for 13 min) at 300 nl/min.

Key parameters for TripleTOF mass spectrometer in SWATH ID and iTRAQ library analysis were: ion spray voltage floating (ISVF) 2300 V, curtain gas (CUR) 30, interface heater temperature (IHT) +125°C, ion source gas 1 13, declustering potential (DP) 100 V. Library for SWATH analysis was created from the same samples by information dependent-aquisition (IDA) method and relative quantitation analysis was done by SWATH method. All methods were run by Analyst TF 1.5 software (Sciex, Redwood City, USA). For IDA parameters, 0.25 s MS survey scan in the mass range 350-1250 mz were followed by 60 MS/MS scans in the mass range of 100-1500 Da (total cycle time 3.302 s). Switching criteria were set to ions greater than mass to charge ratio (m/z) 350 and smaller than 1250 (m/z) with charge state 2-5 and an abundance threshold of more than 120 counts. Former target ions were excluded for 12 s. IDA rolling collision energy (CE) parameters script was used for automatically controlling CE. SWATH quantification analysis parameters were the same as for SWATH ID, with the following exceptions: cycle time 3.332 s and MS parameters set to 15 Da windows with 1 Da overlap between mass range 350-1250 Da followed by 40 MS/MS scans in the mass range of 100-1500 Da.

2.4 Protein identification and quantification

SWATH-MS and iTRAQ library: Protein pilot software version 4.0.8085 (Ab Sciex) was used to analyse MS/MS data searched against the UniProt/Swiss-Prot protein database for protein identification.

Some important settings in the Paragon search algorithm in protein pilot were configured as follows. Sample type: iTRAQ 4plex (peptide labelled), Cys-alkylation: MMTS, Digestion: Trypsin, Instrument: TripleTOF 5600+, Search effort: thorough ID. False discovery rate (FDR) analysis was performed in the Protein pilot and FDR <1% was set for protein identification. Only peptides with 99% confirmation were included in identification and quantification. Shared peptides were excluded from quantification that was performed using ProteinPilot software with straight average of peptides.

As part of the SWATH analysis method, relative protein quantification library, was created using cell samples from this study. Overall library consisted of 13 different samples and 26 data dependent analysis (DDA) runs with same LC gradient and instrument settings which were used for SWATH analyses. Library was created using Protein Pilot® 4.5 (Sciex, Redwood City, USA) and all DDA runs spectra were identified against UniprotKB/SwissProt. Quantification was done by Peak Viewer® and Marker viewer® (Sciex, Redwood City, USA). FDR 1% was used in the library creation and only distinctive peptides were used in the quantification. Retention time calibration was done for all samples using HMR retention time calibration peptides. Five transitions per peptide and 1-15 peptides were used for peak area calculations.

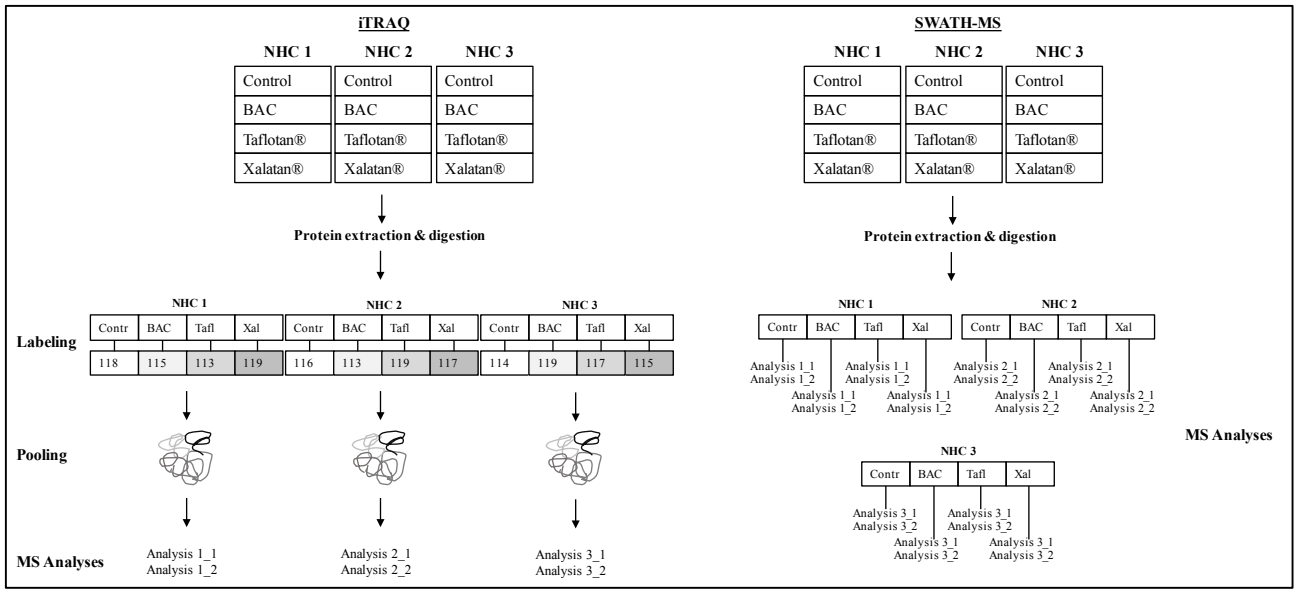

Figure 5. The workflow of the iTRAQ and SWATH-MS, NHC cell experiments. All three sample sets of NHC cells were analyzed from iTRAQ experiments. Due to failed MS analyses, sample set number three from SWATH-MS experiments was excluded from statistical analysis.

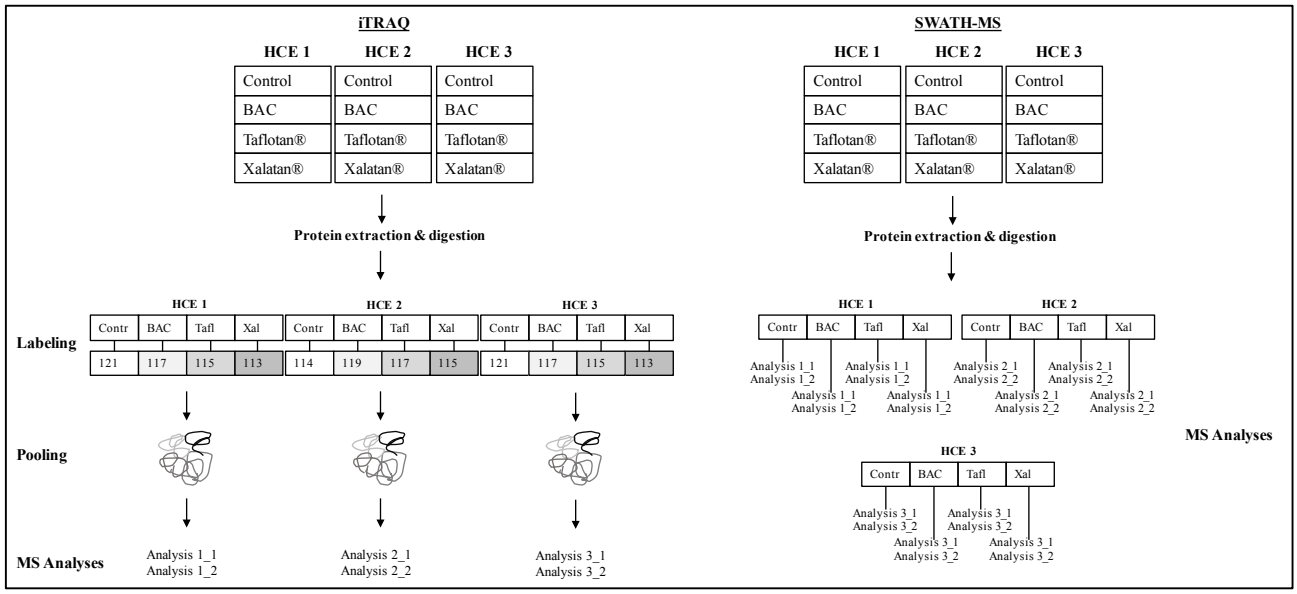

Figure 6. The workflow of the iTRAQ and SWATH-MS, HCE cell experiments. All three sample sets of HCE cells were analyzed from iTRAQ experiments. Due to failed MS analyses, sample set number two from SWATH-MS experiments was excluded from statistical analysis.

3 RESULTS

3.1 Identified proteins

3.1.1 NHC cell experiments

By the SWATH-MS analysis method, 1,917 proteins were identified from each NHC sample and technical replicate. Consequently, the average number of proteins in a sample and in both technical replicates were equal to 1,917. *(Figure 7)* By the iTRAQ analysis method, a total of 1,516 different proteins were identified from all samples and technical replicates. However, only 290 (19 %) of 1,516 proteins were present in all samples and technical replicates. *(Figure 7)* The average number of proteins identified in a sample was 984, and the average number of proteins identified in both technical replicates was 513. *(Figure 7)*

781 common proteins for the SWATH-MS and iTRAQ experiments were identified when the proteins present in at least one technical replicate or sample were taken into account. Furthermore, 1,136 proteins were identified only by the SWATH-MS, and 735 proteins were identified only by the iTRAQ. *(Figure 8A)* However, when only the proteins present in all samples and technical replicates were taken into account, the number of common proteins was 242. In that case, the numbers of proteins identified only by the SWATH-MS or iTRAQ were 1,675 and 48 respectively. *(Figure 8A)* When proteins present in at least one sample versus all samples were taken into account, only 29 % and 12 % of all proteins detected in SWATH-MS and iTRAQ were common for the two methods.

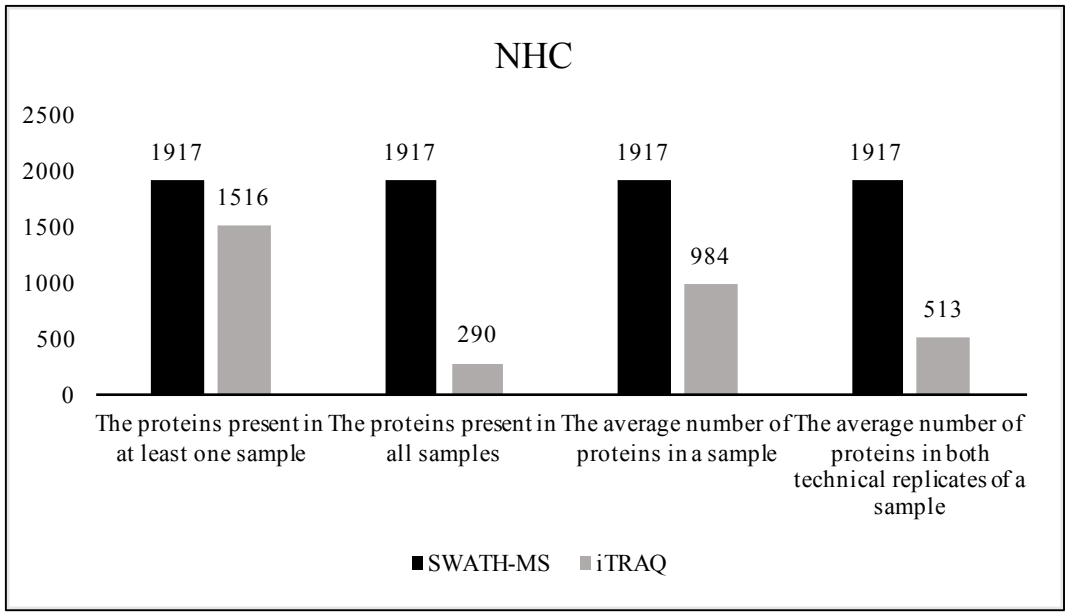

Figure 7. The number of identified proteins by SWATH-MS (black columns) and by iTRAQ (grey columns) in NHC samples. The number of identified proteins are shown when proteins present in at least one sample or in all samples are taken into account. The average number of proteins in a sample and the average number of proteins in both technical replicates of a sample are also shown.

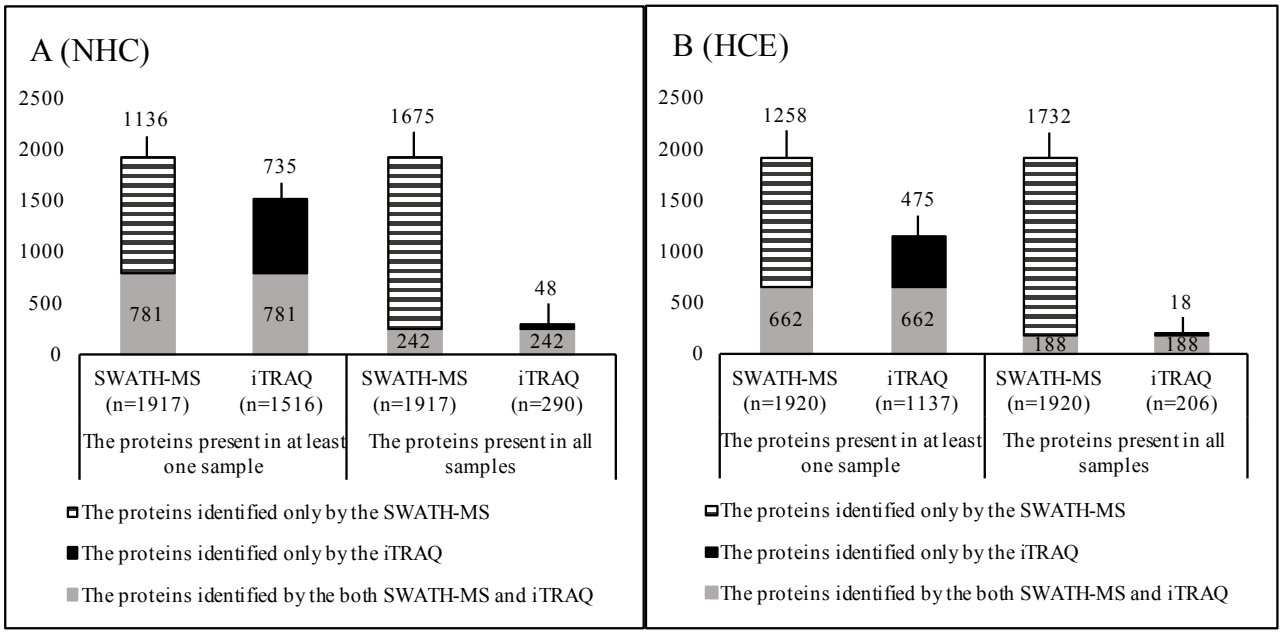

Figure 8. Mass spectrometry method comparison results from the NHC cell experiments (A) and HCE cell experiments (B). Grey columns represent the number of proteins identified by both the SWATH-MS and iTRAQ. Striped columns represent the number of proteins identified only by SWATH-MS whereas black columns represent the number of proteins identified only by iTRAQ.

3.1.2 HCE cell experiments

1,920 proteins were identified from each sample and technical replicate when using SWATH-MS analysis method. Again, the average number of proteins in a sample and in both technical replicates were equal to 1,920. *(Figure 9)* With iTRAQ analysis method, a total number of identified proteins in all samples and technical replicates was 1,137. When only the proteins present in all samples and technical replicates were taken into account, the number dropped to 206 (18 %) proteins. Furthermore, the average numbers of identified proteins in a sample and in both technical replicates were 742 and 381 respectively. *(Figure 9)*

662 overlapping proteins were identified in the SWATH-MS and iTRAQ experiments when the proteins present in at least one sample were taken into account. In that case, 1,258 proteins were identified only by the SWATH-MS whereas 475 proteins were identified only by the iTRAQ. *(Figure 8B)* However, only 188 common proteins for the SWATH-MS and iTRAQ were identified when the proteins present in all samples and technical replicates were taken into account. Furthermore, in that case, 1,732 proteins were identified only by the SWATH-MS, and 18 proteins were identified only by the iTRAQ. *(Figure 8B)* Thus, only 18 % (proteins present in at least one sample) and 10 % (proteins present in all samples) from all proteins detected in iTRAQ and SWATH-MS were common for the two methods.

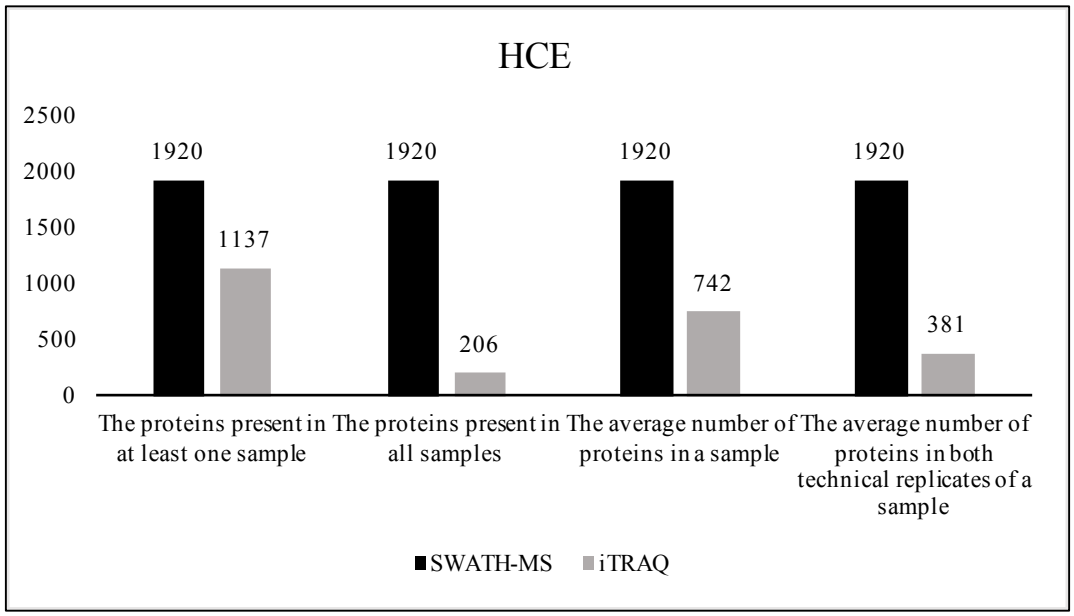

Figure 9. The number of identified proteins by SWATH-MS (black columns) and by iTRAQ (grey columns) in HCE samples. The number of identified proteins are shown when proteins present in at least one sample or in all samples were taken into account. Furthermore, the average number of

proteins in a sample and the average number of proteins in both technical replicates of a sample are shown.

3.2 The quantification reproducibility

The relative standard deviation (RSD) was used to assess the quantification reproducibility. With regard to the SWATH-MS experiments, 1,917 proteins from NHC data and 1,920 proteins from HCE data were detected in all samples and technical replicates. Furthermore, there were eight samples in both, NHC and HCE cell experiments, thus, in total 15,336 and 15,360 measured results from the samples were taken into account respectively. With regard to the iTRAQ data, 290 and 206 proteins were detected in all samples and technical replicates in NHC cell and HCE cell experiments respectively. There were nine samples in each cell experiment, thus, 2,610 measured results from the samples from NHC cell experiments and 1,854 measured results from the samples from HCE cell experiments were included.

3.2.1 NHC cell experiments

A total of 39 % (6,038) of measured results had RSD lower than 10 % in the SWATH-MS data, whereas a total of 66 % (1,729) of measured results had RSD lower than 10 % in the iTRAQ data. (*Figure 10*) The range between samples was 12-59 % (233-1,122) in the SWATH-MS data and 59-70 % (170-203) in the iTRAQ data. The numbers of measured results with RSD below 20 % were 55 % (8,433) and 88 % (2,297) for the SWATH-MS and iTRAQ respectively. (*Figure 10*) The ranges between samples were 26-73 % (492-1,393) and 83-93 % (240-271) for SWATH-MS and iTRAQ respectively. Mean RSD in the SWATH-MS data was 31 % and in iTRAQ data 10 %.

3.2.2 HCE cell experiments

With regard to the SWATH-MS data, a total of 54 % (8,294) of measured results had RSD lower than 10 %, and with regard to the iTRAQ data, a total of 62 % (1,151) of measured results had RSD lower than 10%. (*Figure 10*) The range between the samples was 30-65 % (571-1,332) in SWATH-MS data and 53-66 % (109-135) in the iTRAQ data. In the SWATH-MS data, a total of 72 % (11,114) measured results had RSD lower than 20 %, whereas in the iTRAQ data the corresponding number was 85 % (1,576). (*Figure 10*) The range between the samples was 57-82 % (1,086-1,576)

in the SWATH-MS data and 73-90 % (151-185) in the iTRAQ data. Mean RSD in the SWATH-MS data was 19 % whereas in the iTRAQ data it was 11 %.

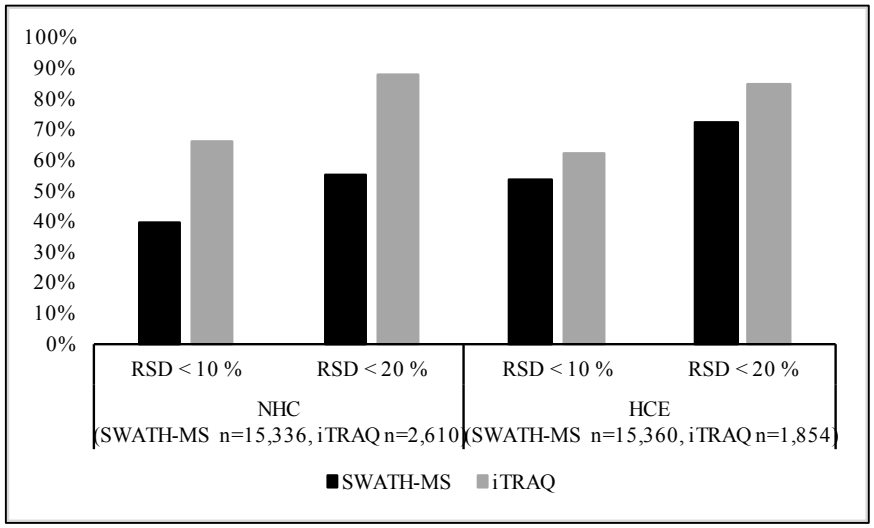

Figure 10. The bar graph shows the percentage values of RSDs below 10 % and 20 % across all samples. Results from the NHC cell experiments are shown on the left and results from the HCE cell experiments are shown on the right. Black columns represent results from the SWATH-MS experiments and grey columns represent results from the iTRAQ experiments.

3.3 Expression level bias and correlation of the expression levels between SWATH-MS and iTRAQ

Next, we wanted to examine how precisely SWATH-MS and iTRAQ could quantify protein levels, and how similar the expression levels were between the SWATH-MS and iTRAQ. First, we evaluated the number of under- and over expressed proteins in each sample, and compared the results between the analysis methods. Second, we evaluated how much the expression levels differed from each other between SWATH-MS and iTRAQ. Third, we evaluated how similar the directions of expression level fold changes were between SWATH-MS and iTRAQ. Finally, we evaluated was there any linear relationship in expression levels between SWATH-MS and iTRAQ.

Under/over expression analysis and direction of expression level fold change analysis: 242 proteins from NHC cell experiments and 188 proteins from HCE cell experiments were detected in all samples and technical replicates and identified by both analysis methods. Thus, a total of 1,452 and

1,128 expression level results were included in these analyses from NHC and HCE cell experiments respectively.

Expression level difference and linear relationship analyses: In the NHC cell experiments, 781 proteins were detected in at least one sample or technical replicate and identified by both analysis methods. In the HCE cell experiments, the corresponding number was 662. We included all these proteins in above-mentioned analyses, and thus, a total of 3,312 and 2,754 measured expression level results were included from NHC and HCE cell experiments respectively.

3.3.1 The trends of under- and over expressed proteins

NHC cells: With regard to the SWATH-MS data, in a sample on the average 51 % (124) proteins were under expressed, and on the average 49 % (119) proteins were over expressed. Correspondingly, in the iTRAQ data the average number of under expressed proteins in a sample was 41 % (99) and the average number of over expressed proteins in a sample was 59 % (142). As a matter of fact, although the percentage values are quite equal between the two analysis methods, there was comparatively more variation in the iTRAQ data than in the SWATH-MS data: standard deviations were 8 and 96 for SWATH-MS and iTRAQ respectively. Therefore, the percentage values of under- and over expressed proteins are shown in *Figure 11* separately for every sample. As can be seen from bar graph, while the SWATH-MS results are quite similar between samples, there is vast difference between the iTRAQ results (*Figure 11*).

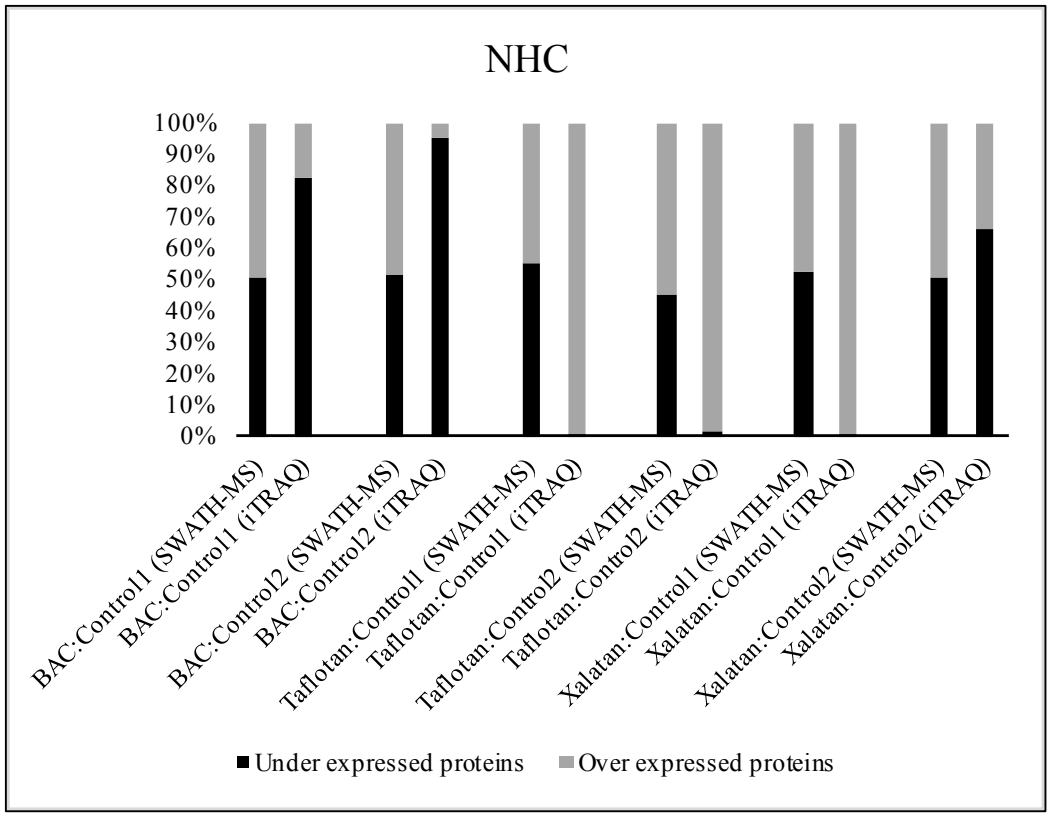

Figure 11. The percentage values of under- and over expressed proteins in each sample. Results from NHC cell experiments.

HCE cells: In the SWATH-MS data, the average number of under expressed proteins in a sample was 54 % (101), and the average number of over expressed proteins in a sample was 46 % (87). In the iTRAQ data, the numbers were 67 % (126) and 33 % (63) respectively. Standard deviation for SWATH-MS results was 9, whereas for iTRAQ results it was 78. Likewise in the NHC cell data, obviously there was also more variation in the iTRAQ data than in the SWATH-MS data. *Figure 12* shows the percentage values of under- and over expressed proteins for each sample. Again, it can be seen from bar graph that there are vast differences between samples in iTRAQ while the SWATH-MS results are quite similar to each other (*Figure 12*).

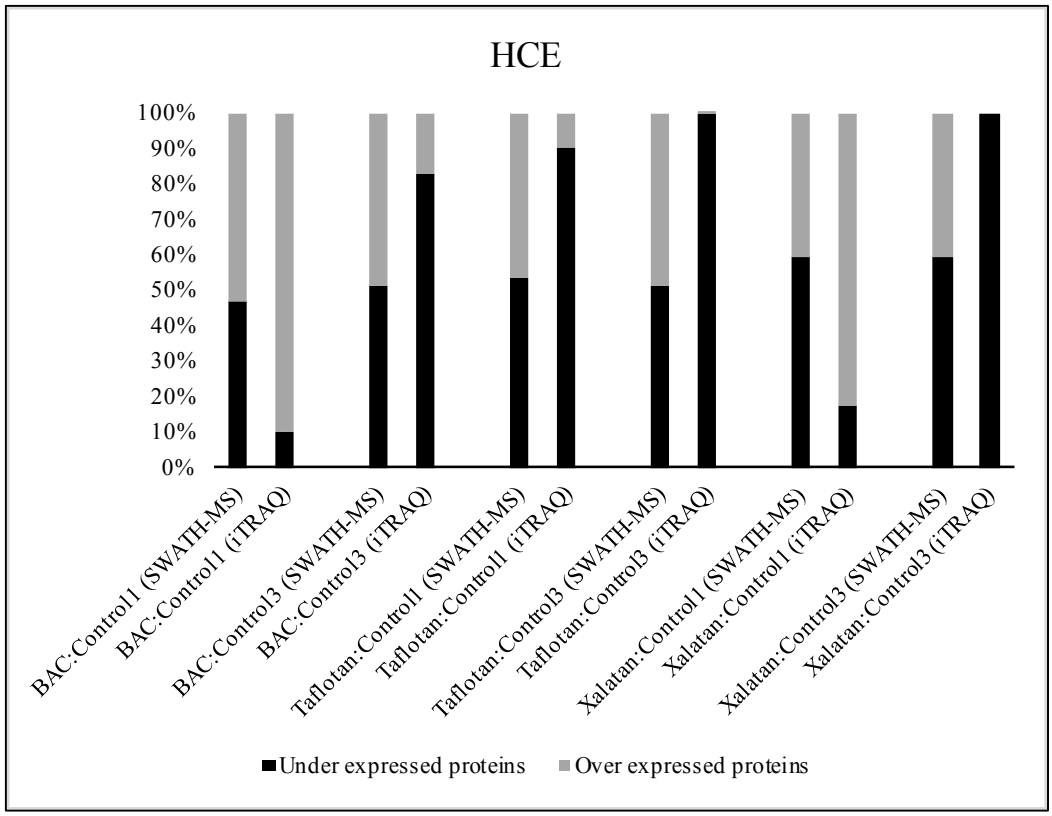

Figure 12. The percentage values of under and over expressed proteins in each sample. Results from HCE cell experiments.

3.3.2 Correlation of the expression levels between SWATH-MS and iTRAQ

SWATH-MS and iTRAQ showed congruent direction of protein expression fold changes in 51 % of the results in NHC cell data, and in 53 % of the results in HCE cell data. There was only a slight difference in expression levels of proteins between SWATH-MS and iTRAQ. In the NHC cell data, 95 % of the expression level results differed from each other less than one, and the corresponding number in the HCE cell data was 97 %. This outcome was seen regularly across all samples: the standard deviation of expression level results which differed less than one between SWATH-MS and iTRAQ was 3 % in both NHC and HCE cell data. There was no linear relationship between the expression levels of proteins from the iTRAQ and SWATH-MS experiments in either cell line data (R^2=0,000035-0,05765), i.e. the expression level results didn't correlate between the analysis methods.

4 DISCUSSION

In this *in vitro* study, we compared two different mass spectrometric methods; the SWATH-MS and iTRAQ. Only two previous studies have compared the specific methods mentioned above (53,84). First we evaluated the number of identified proteins by two different methods. Due to nature of SWATH-MS analysis, the number of identified proteins depends on the coverage of spectral library (42). From a few hundred to few thousand proteins have been identified by SWATH-MS in previous studies (44,52,53,84). In our study, we identified 1,920 and 1,917 proteins which were all detected in all samples and technical replicates. Thus, SWATH-MS proved to provide reproducible identification across all samples. By contrast, we identified 1,516 and 1,137 proteins by iTRAQ. However, only less than a fifth of those were detected in all samples and technical replicates. Similar to our iTRAQ results, in previous iTRAQ studies, the number of identified proteins ranged also from few hundred to few thousand (64-66). In two previous studies comparing SWATH-MS and iTRAQ, more proteins have been identified by iTRAQ (53,84). Our results differ from those since we identified more proteins by SWATH-MS. The difference was emphasized especially when observing proteins present in all samples and technical replicates (*Figure 8*).

In order to evaluate the quantification reproducibility, we assessed relative standard deviations (RSDs, also known as coefficient of variation = CV). Comparison between the methods indicated that iTRAQ would have better quantification reproducibility. iTRAQ RSD values were relatively more often below 10 % or 20 % when compared to SWATH-MS RSD values. However, the number of analyzed results from the SWATH-MS data was considerably higher: 6-fold in NHC cell data and 8-fold in HCE cell data. With regard to the SWATH-MS, the results indicated more reproducible quantification in the HCE cell experiments than in the NHC cell experiments. By contrast, the results from the iTRAQ data were more consistent between the cell lines. With regard to iTRAQ, the variance of expression level results has been proved to be higher for low intensity peptides. Moreover, the effect has been irrespective of labeling kit, mass spectrometric instrumentation, or analytical packages (64-66). With regard to SWATH-MS, variance has been proved to be comparable between high and low intensities, however, variance has been smaller on samples with higher protein amounts (44,52). In our study, we didn't evaluate dependence between RSD and protein intensities or sample load.

There are several ways to estimate the correlation of the protein quantification results between SWATH-MS and iTRAQ. To begin with, we evaluated the number of identified proteins by both analysis methods: only 10-29 % of all proteins were common for the two methods, depending on included proteins (present in at least one or in all samples). Our results were not as good as in previous studies, in which 37-51 % of all identified proteins were detected by both methods (53,84). Next, the similarities in direction of protein expression fold changes was evaluated between the methods. Bourassa et al. (2015) showed that SWATH-MS and iTRAQ agreed on the direction of the fold changes 92-98 % depending on used threshold (53). Our results didn't achieve that level: SWATH-MS and iTRAQ agreed on the direction only on 51 % in the NHC cell experiments and 53 % in the HCE cell experiments. Across all samples in the SWATH-MS data, approximately half of the proteins were under expressed and the other half over expressed, while in the iTRAQ data, most of the proteins in a sample were either under- or over expressed. (*Figure 11 and 12*) Hence, the foregoing is the main reason for poor correlation of the direction of fold changes between the methods.

To further examine the correlation of expression levels results between SWATH-MS and iTRAQ, we included all proteins present in at least one sample or technical replicate in next analyses. Only 5 % of proteins in the NHC cell data and 3 % of proteins in the HCE cell data yielded expression levels which differed more than one from each other between the methods. Thus, the expression levels of proteins were quite consistent between the two methods. Finally, we evaluated if there was linear relationship in expression levels between the methods, which was not found. In theory, SWATH-MS and iTRAQ should have yielded similar expression level results for proteins since samples were exposed and processed similarly (apart from iTRAQ labeling) prior to mass spectrometric analysis. On one hand, the lack of linear relationship may be since the expression levels were small enough to fit in the error limits of the methods. On the other hand, those maximum 5 % of the proteins which differed more than one from each other between the analysis methods might have differed enough to eliminate linearity.

As discussed in introduction, underestimation of protein expression fold changes has proved to be the main challenge with the iTRAQ (54). Ratios are compressed towards one, thus, observed fold changes are reported smaller than true fold changes are. The effect is more pronounced with the larger fold changes. (37,65,66) However, the directions of fold changes have proved to be correct (54). In two previous studies, a small underestimation effect has been seen also with SWATH-MS (44,53). 1:1 ratios were quantified with high accuracy, and larger fold changes were more

underestimated as seen in iTRAQ studies too (44). However, the underestimation effect of iTRAQ is more eminent (53).

In conclusion, we compared two proteomic methods in this *in vitro* study. SWATH-MS proved to be superior to iTRAQ especially regarding the identification rate and reproducible quantification. Based on the results of this study and previous studies, we would recommend using primarily SWATH-MS in corresponding analyses in future.

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